IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	28062
Docket No.:	ZR05.012
Applicant:	David GERSHON
Application No.:	10/561,323
Filed:	December 19, 2005
For:	MICROBICIDAL, PROPHYLACTIC AND THERAPEUTIC EFFECT OF CTC-96 ON PAPILLOMA VIRUSES
Group Art Unit:	1617
Examiner:	Zarek, Paul E.
Confirmation No.:	5879

Commissioner for Patents (via EFS)
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO RULE 132

David Gershon, hereby solemnly and sincerely declares that:

1. He is the inventor of the invention described and claimed in the above identified application and is familiar with the application and the prosecution history thereof:

2. He cause the experiments described herein below to be carried out and the data presented is the data obtained from the described experiments:

CTC-96" in saline was incubated with HPV-11 prior to infection of human neonatal foreskin fragments. The fragments were then grafted onto SCID mice. The animals were monitored weekly. They were weighed at the time of grafting, and every other week during the 12 weeks of *the* experiment. There was no effect of HPV-11 treatment by CTC-96" on the weight of the mice. The animals were sacrificed by cervical dislocation 12 weeks after graft implantation. Length, width, and height of the graft were measured and recorded. A composite geometric mean diameter (cGMD) of the grafts was calculated for each mouse. The grafts were then removed and analyzed by histology, immunocytochemistry and RT-PCR.

Graft evaluation by immunocytochemistry utilized anti-common Papillomavirus antigen.

Explanted grafts were homogenized and total RNA was extracted. HPV-11 viral cDNA was generated by nested RTPCR..

Effect of CTC-96" on Graft Size

Comparison of the cGMDs in the treatment groups establishes that regardless of the CTC-96M concentration there was a small but significant effect on the infectivity of HPV-11 when compared to the control (no CTC-98Tm) (Table 1).

Table 1: Composite Geometric Mean Diameters (cGMD) of the Grafts (mm)

Treatment		N	Mean t SD		Median'
CTC-96m	0 %	9	2.58 ±	0.80 8	2.59
C1C96TM	0.05	8	2.02 ±	0.17 9 -	2.01
CTC-96	0.2%	6	1.86 ±	0.19. 6	1.88
CTC-96M	1%	9	1.95 #	0.13 9	1.94

Effect of CTC-96[111] and HPV-11 Treatment on Graft Histology

Table 2 shows the results of the histologic examination of the grafts for the presence of HPV. Presence of HPV was defined by the presence of two out of three of the following features: acanthosis (an increase in the thickness of the stratum spinosum of the epidermis) koilocytosis (perinuclear vacuolation), or parakeratosis (persistence of the nuclei in the cells of the stratum corneum of the epidermis). As the CTC-98'm concentration increases the percentages of grafts containing HPV decreases, with none positive at the two higher concentrations. At these two highest CTC-96m concentrations (0.2% and 1%) the vast majority of the grafts displayed a fibrous tissue or a foreign body reaction and were thus classified as non-interpretable. Two explanations are possible for the high number of foreign body reactions present in the grafts infected with the HPV-11 treated with the two highest

concentrations of CTC-96M. The first is that CTC-96M completely inactivated HPV-11. Hence the grafts were not infected, and as a consequence were more likely to be eliminated by a foreign body reaction. The alternate explanation is that CTC-96m was 'toxic to the graft itself. This is unlikely since CTC-96m has been used *in vitro* and *in vivo* in intravaginal preparations without associated toxicities at the concentrations used, and at the lowest concentration of CTC-96m (0.05%), the number of grafts with a foreign body reaction was not different than that in the control group, thus toxicity would be unlikely to account for the lower number of HPV positive grafts (P 0.0 15; by Fisher exact test). Since CTC-96" has a clear virucidal action at the lowest concentration (see Table 4), it is reasonable to assume that this virucidal action is present at higher concentrations independent of the presence of a cytotoxic effect:

Table 2 presents the results when grafts exhibiting a foreign body reaction or fibrous tissue were counted as negative for HPV. The conclusion that CTC-96" has virucidal activity against HPV-11 remains identical to that of the previous analysis, but with more statistical significance because of the greater number of observations.

Table 2: Histology CTC-96[174] and HPV-11 Treated on Grafts *

Treatment	Negative	Positive	Totals
CTC-96 ™ 0 %	6	11 (64.7%)	17
CTC-96[1"] 0.05%	11	4 (26.7%)	15
CTC-96" 0.2%	12	0 (0%)	12
CTC-96 ™ 1 %	16	0 (0%)	16

* in this analysis, observations counted as negative include grafts that were read as negative for HPV as well as grafts that displayed a foreign body reaction or some fibrous tissue

In Table 3 grafts exhibiting foreign body reaction or fibrous tissue are considered negative for HPV. The conclusion that CTC-96" has virucidal activity against HPV-1 is unchanged.

Table 3 - Immunocytochemistry of CTC-96[1™] and HPV-11 Treated on Grafts *

Treatment	Negative	Positive		Totals
CTC-96™ 0 %	10	7	(41.2%)	17
CTC-96™ 0.05%	14	2	(12.5%)	16
CTC-96™ 0.2%	12		(0%)	12
CTC-96™ 1%	17	0	(0%)	17

*. Histology of the grafts was reviewed for the presence of HPV as measured by immunocytochemistry using anti-common Papillomavirus antigen[4]. In this analysis, observations counted as negative include grafts that were read as negative for HPV as well as grafts that displayed a foreign body reaction or some fibrous tissue

Effect of CTC-96 on Graft HPV RT-PCR

Table 4 presents the results and analysis of the HPV RT-PCR, which was performed by extracting total RNA from homogenized, explanted grafts and generating HPV-11 viral cDNA by nested RT-PCR. Using this assay, there is a strong and clear dose-response effect. Only with

- the highest dose of CTC-96™ is there complete obliteration of viral transcriptional activity. In contrast with the histology and immunocytochemistry results where the two highest doses of CTC-96™ did not show evidence of the presence of HPV, with the RT-PCR, HPV cDNA was detected in two grafts from the CTC-96™ 0.2% group.

Table 4 RT-PCR of CTC-96 and HPV-11 Treated Grafts-

Treatment	HPV-11 Negative	HPV-11 Positive		Non-interpretable	Totals
CTC-96™ 0%	4	13	(76.5%)	0	17
CTC-96™ 0.05%	7	9	(56.2%)	0	16.
CTC-96 0.2%	5	2.	(28.6%)	5	12
CTC-96 1%	15		(0%)		17

These samples were not interpretable because both the HPV and P-actin messages were not detected.

The RT-PCR results support a virucidal rather than a toxic effect of CTC-96".

CONCLUSIONS

The treatment of HPV- 11 with CTC-96 had an. inhibitory effect on the infectivity of the virus as measured by graft size *in* the human xenograft SCID mouse model with the three tested concentrations of CTC-96 leading to a drastic inhibitory effect on graft size when compared to the control group. Analysis of the presence of HPV by histology, immunocytochemistry, and RT-PCR, demonstrated a clear dose-response effect. The virucidal effect of the lowest concentrations of CTC-96" was only partial while the highest concentration of CTC-96" (1%) appeared completely virucidal. CTC-96"" did not exert its action by direct toxicity on the grafts. As analyzed by RT-PCR, the vast majority of the grafts were viable at the end of the experiment despite lacking evidence of HPVIranscription. CTC-96' had no effect on the animals' mortality or weight gain. CTC-96' did not stimulate viral or cellular replication.

Evaluation of CTC-96^{7*4} Therapeutic Activity on Human Condylomas Induced by HPV-11 in the HPV-11-Infected External Human SCID Mouse Model

Two different dose levels of CTC-96' (1 %, 0.1$^{1)}$/0) in an ointment formulation and the vehicle alone were evaluated. SCID mice were grafted on each side of the dorsum with an HPV-11infected foreskin fragment. The HPV-11-infected grafts were left to grow for 6 weeks before treatment was started and continued for 6 weeks. During the treatment phase, the drug was administered thrice a week, directly on the graft. Graft length, width, and height were measured every two weeks during the treatment phase. At the end of the study, the animals were sacrificed. The grafts were measured, recovered, and processed for histology. HPV infection in tissue sections is defined by the presence of two out of three of the following features: acanthosis, koilocytosis, or parakeratosis.

Effect of CTC-96^{7*} on Graft Size

The Graft Size Growth (GSG) index was our primary endpoint in this evaluation of CTC-96^{7n}. Table 5 provides the summary statistics of this measurement. There is a gradual

increase in the mean GSG with higher CTC-96" concentration, which would suggest, that CTC-96" stimulates the growth of Papillomavirus-infected tissue. However, there is also a marked increase in the variance of the GSG in the high dose 'CTC-96[11o] group.

Table 5: Graft Size Growth (%)

Treatment	N	Means ± SD	Median
CTC-96[74] ti %	15	57.50 ± 48.59	50.65
CTC-96 0.1%	14	64.53 ± 41.92	60.51
CTC-96 [TM] 1%	16	91.39 t 127.84	52.03

In our analysis, to measure graft size growth we used a composite index that summarizes for each mouse the growth of the two grafts borne by the animal, each from a different foreskin donor. If instead we look at the growth of each individual graft, we can see that the means, medians, or variances of the graft size growth are more alike among the three treatment groups as shown in Fig. 1. The ANOVA fails to show a treatment effect on the growth of the individual grafts.

CTC-96" has been found to be virucidal against Human Papilloma Virus (HPV) Type 11[5] with no detectable *effect* on the growth of HPV-11Anduced papillomas [B]. CTC-96" neither decreased nor increased the growth of HPV-11-infected human papillomas. Given the virucidal activity of CTC-96"", a series of experiments was performed to evaluate the prophylactic activity of the compound under conditions resembling that of the use of a topical microbicide. When using a topical microbicide the target organ is first exposed to the microbicide before exposure to the virus. Various concentrations of CTC-96" were evaluated in a model of HPV-11-infected human xenograft in the SC1D mouse. The human grafts were exposed to CTC-96"" for 1 hour prior to exposure to'HPV- 11 and engraftment. Because CTC-96[1"] is virucidal, it was washed off the foreskin fragments before exposure to the virus. The HPV-11-infected grafts were allowed to grow for 12 weeks. After 12 weeks, the animals were sacrificed and the grafts recovered, measured and processed. Graft size, expressed as the composite geometric mean diameter of the two grafts borne by the animal, was the primary endpoint. Histology of the grafts was examined for the presence of HPV.

Grafts were also processed for detection of HPV-11 mRNAs transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR)..

Results are in agreement with a dramatic and highly statistically significant microbicidal effect of CTC-96" as shown in Fig. 2. Graft size in the control group was significantly larger than that in each of *the* groups in which CTC-96" was used. However, the differences were not statistically significant between the groups in which the foreskin fragments were treated with CTC-96[1]".

Table 6 summarizes the histology results for the presence of HPV in the grafts. As the CTC96T" concentration increased, the number of grafts displaying either fibrous tissue or a foreign body reaction also increased. Grafts that are not infected by HPV proliferate poorly and tend to be eliminated by the mouse's host defenses as a foreign body. The residual fibrous tissue is a scar. In order to analyze the results properly, grafts that were truly negative (graft showing a healthy human epithelium) as well as those showing a foreign body reaction or fibrous tissue were counted as negative for HPV. These results also suggest that CTC-96" has a microbicidal effect on HPV-11.

Table 6: Histology Results for the Presence of HPV

Treatment Group	Histo ogy				
	Negative		Positive .		Taal.
CTC-96' 0%	2	10.5%	17	89.5%	- 19
CTC-96 ™ 0.05%	7	30%.	16	73%	23
CTC-96" 0.2%	14	67%		33%	21
CTC-96[7]" 1%	22 •	. 100%	0	. 0%	22

Table 7 summarizes the immunocytochemistry results for the presence of HPV capsid antigen in the grafts. As the concentration of CTC-96" increases, the number of grafts displaying either fibrous tissue or a foreign body reaction also increases. Therefore, the table was constructed such that the grafts demonstrating fibrous tissue or a foreign body reaction were counted as negative. Again, a strong microbicidal effect of CTC-96" is observed. •

Table 7: Modified immunocytochemistry Results for the Presence of HPV

Treatment Group	Immunocytochemistry				
	Negative		Positive		Total
CTC-96" 0%	3	16%	16	84%	19
CTC-96 ™ 0.05%	11	48%	12	52%	23
CTC96' 02%	15	71%	6	29%	21
CTC-96' 1%	22	100%	0	0%	22

Table 8 summarizes the results of the analysis of the grafts for HPV-11 expression. These results indicate that 22% of the grafts still showed viral expression with the highest concentration of CTC-96". This demonstrates that viral infection is not necessarily accompanied by tissue proliferation. Some of the grafts that demonstrated the presence of fibrous tissue or foreign body reaction also contained HPV mRNA.

Table 8: RT-PCR Results for the Presence of HPV

Treatment Group	RT-PCR				
	Negative		Positive		Total
CTC-96[114] 0%	5	22%	18	78%	23
CTC-96 ™ 0.05%	9	39%	14	61%	23
CTC-96 ™ 0.2%	17	81%	4	19%	21
CTC-96 ™ 1 %	14 -1	78%	4	22%	18

In conclusion, CTC-96[1]m was shown to have a strong effect on HPV-11 when used under conditions simulating the natural infection. Although CTC-96[1]m did not completely block infection, even at the, highest concentration (1%), the clinical markers of infection were absent (graft proliferation, signs of HPV infection by histology and immunocytochemistry).

Inactivation of Bovine Papillomavirus type 1 (BPV-1) by CTC-96"'

Experiments were performed to determine if CTC7 96 can inactivate the ability of bovine papillomavirus type 1 (BPV-1) to morphologically transform C1 27 mouse epithelial cells in culture. Cell-free stocks of BPV-1 were treated with CTC-96 or placebo.

in order to detect the transforming ability of BPV-1, sub-confluent cultures of Cl27 mouse cells are infected with a standardized inoculate of BPV-1. For the positive controls, stock virus was added to the culture medium present on the cells. The presence of morphologically transformed foci was counted after 2 weeks and then again at 3 weeks. Controls included non-infected cells (with or without drug) and untreated BPV- 1.

Bovine Papillomavirus Type 1 (BPV-1) was mixed with CTC-96", incubated for 10 minutes at 37°C and then added to the cells. It is clear that CTC-96"" can inhibit the appearance of bovine Papillomavirus type 1 (BPV-1) induced transformation of Cl27 mouse epithelial cells in culture (Table 9).

Table 9: CTC-96^{70w} Inhibition of Bovine Papiliomavirus Type 1 (BPV-1) Transformation of C127 Mouse Epithelial Cells in Culture: Exposure of Virus to Drug Prior to Infection.

Treatment	CTC-98Tm (mg/m1)	Number of Transformed Foci at 3 weeks	
PBS alone	-	0	0
BPV-1 alone		96	105
CTC-96m	0.1	68	80
CTC-96 ^{TM}	0.2	80	82
CTC-98 ^{TM}	0.5	25	40

To further evaluate the time course of interaction between CTC-96^{114} and PapillomaviruS the following experiment was performed. Virus was put on C127 Mouse epithelial cells in culture at time 0 and incubated for 5 hours. Medium was then removed and replaced with medium plus drug. Cells,were then re-fed every 2 days and the experiments counted on day 12. The results shown in Table 10 suggest an effect of the compound on the virus in the infected cells, and not on extracellular virus.

Table 10: CTC-96· Inhibition of Bovine Papillomavirus Typed (BPV-1) Transformation of C127 Mouse Epithelial Cells in Culture: Exposure of Cells Pretreated with Virus to Drug.

Treatnient	CTC-96™ (P9/ml)	Number of Transformed Foci			
		Exp. 1		Exp. 2	
PBS alone		0			
BPV-1 alone		81·	72	41	43
PlaCebo	-	77	58 •	20	21 •
CTC-96 ™	5	3	-2	3	3

To further clarify the dynamics of CTC-96" Papillomavirus interaction an experiment was performed to combine exposure of virus to the drug prior to cellular infection and exposure of infected cells to the drug. For this purpose BPV-1 was incubated with either CTC-96" or placebo, diluted and then added to Cl27 Mouse Epithelial Cells in culture with the addition of CTC-96™ or placebo (Table 11). Addition of 10pg/mlor 20).tg/m1CTC-96" to the cell cultures was toxic to the cells. Addition of 51.1g/mlCTC-96" effectively suppressed all focus formation. Pre-treatment with 5012g/mlCTC-96" but not placebo halved the foci number in the normal medium post-treatment control. Thus a small microbicidal effect is probable even at these low concentrations of CTC-96". The main activity at these concentrations, however, is post- infection. However, pretreatment of the virus (microbicidal effect) also caused 50-60% inhibition of the virus.

Table 11: CTC-96M. Inhibition of Bovine Papillomavirus Type 1 (BPV-1) Transformation of C127 Mouse Epithelial Cells in Culture: Pretreatment and Post-treatment combined

Pre-treatment		Pest-treatment	Number of Transformed Foci			
			Experiment 3-1		Experiment 3-2	
Mock Inoculum		Normal medium	0	0	0	0
BPV		Normal medium	163	156	183	148
Placebo	BPV	normal medium	175	117	155	149
Placebo	BPV	placebo	150	130	- 133	122
Placebo	BPV	5gg/mlCTC-96	0	0	0	0
501.1g/m1 OTC-96	BPV	normal medium .	71	46 '	46	41
501,1g/ml CTC-96	BPV	placebo .	65	61	39	62
5014/m1 CTC-96	BPV	51.1.g/mlCTC-96	0	0	0	0

I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and I understand that any willful false statements and the like are punishable by fine or imprisonment or both and may jeopardize the validity of the application or any patent issuing thereon.

8/30/10
Date

David Gershon
David Gershon